AET AS A RADIOPROTECTIVE AGENT AT THE CELLULAR LEVEL[1]

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Many agents have been tested for their possible protective effect against the damaging and lethal action of ionizing radiations (Rugh, 1953a, 1958; Hollaender and Doudney, 1954; Thomson, 1962). Among the most effective agents for the mature mammal are AET (S,2-aminoethylisothiourea-Di-HBr), MEG (2-mercaptoethylguanadine), MEA (B-mercaptoethylamine) and cysteine HCl (Doherty and Burnett, 1955, 1961; Stern, 1956; Doherty *et al.*, 1957; Doherty and Shapira, 1958; Maisin and Doherty, 1960; Maisin and Popp, 1960; Maisin, 1961; Mundy *et al.*, 1961; Dacquisto *et al.*, 1961; Blouin and Overman, 1962; Melville and Leffingwell, 1962; Ehling and Doherty, 1962; Hanna and Colclough, 1963; Mittler, 1963). AET has also been shown to be protective against the effects of the so-called radiomimetic agents (Asano *et al.*, 1962, 1963). In every case the drug used, to be effective, had to be administered prior to exposure to the ionizing radiations. Usually such agents, injected after irradiation, were deleterious. Only spleen and bone marrow homogenates appear to have any protective value when administered after exposure (Bacq and Alexander, 1961).

The usual explanation for the so-called protective action of any of these agents is that they somehow aid in hematopoietic recovery (Dickens and Shapiro, 1961; Colclough and Hanna, 1963) or cause hypoxia (Hanna and Colclough, 1963). How protection is actually accomplished is not at all clear.

In order to understand better the mechanism of protection, it seemed wise to test the most effective agent, AET, at the cellular level. For this study the unfertilized egg of the sea urchin, *Arabacia punctulata*, was used. Thus, any irradiation and possible radioprotective action of AET on the haploid cells would be reflected in the early cleavage stages, as well as in early organogenesis. This is a report of the findings.

Materials and Method

The eggs of *Arbacia punctulata* were obtained from 5 or 6 mature females by cutting away the Aristotle's lantern from the oral region and inverting them in stender dishes of filtered sea water, and allowing them to shed naturally through the five gonopores. Such eggs were washed in two changes of filtered sea water, 15 minutes apart, and allowed to settle. When the eggs were to be subjected to AET, two times the desired concentration of the agent was used, added to an equal volume of egg suspension, resulting in the proper concentration of AET.

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All eggs were fertilized simultaneously by a sperm suspension made from a single sea urchin.

The radiation facilities consisted of the cesium-137 paired sources available at the Marine Biological Laboratory, Woods Hole, Mass. The eggs were suspended midway between the two sources of these gamma rays at a distance of 5.9 cm. from each source and a dose rate of 5000 r/min. After some exploratory tests a total exposure of 50,000 r was chosen, delivered in 10 minutes.

The AET was obtained from Dr. D. G. Doherty of Oak Ridge National Laboratory, and was identical with that used by him so successfully with mammals (Doherty *et al.*, 1957), and was kept in a desiccator. In order to convert the AET into the effective MEG, it was first dissolved in 10 cc. of phosphate buffer at pH 7.4, before diluting with 490 cc. of filtered sea water, resulting in pH range from 7.3 to 7.6. This is known to be optimum for the normal development of *Arbacia* (Harvey, 1957). The concentration of AET to be used was empirically determined, being the threshold level just below toxicity (see below). The solution thus provided was largely MEG which had to be used within 30+ minutes, before its oxidation degradation to the disulfide.

All studies were made at the laboratory temperature, which ranged from 21.5° to 23.0° C., but was uniform for any single set of experimental variables. *Arbacia* samples were fixed in 10% formalin in filtered sea water at 20-minute intervals, beginning one hour after fertilization when the controls normally show a high percentage of cleavage. Percentages were based upon a minimum of 200 cell counts, the data presented here (Table 1) being on 400 cell counts.

When eggs were subjected to AET this was done for a minimum of 10 minutes prior to irradiation in order to bring this agent into equilibrium with the egg substance. When *Arbacia* eggs were returned to sea water from the AET solution they were diluted with approximately 100 times the volume of filtered sea water.

Experimental Data

Exploratory experiments indicated that exposures of the unfertilized eggs of *Arbacia* to 5000 r or more resulted in cleavage delay and 50,000 r still allowed 11% to cleave, after considerable delay over the controls. Some 18% showed anomalies during the early cleavages. None achieved the pluteus stage, nor even gastrulation. Thus, if AET were able to "protect" such irradiated eggs this would be revealed in a shortening of the time from fertilization to the first cleavage, an increase in the percentage of cleavage within a given period, reduction in the incidence of anomalies and/or effects on development past gastrulation. This exposure of 50,000 r did not render the eggs unfertilizable by normal sperm and they readily formed fertilization membranes. There was some evidence that these membranes were not fully elevated, and that the perivitelline space was not as wide as in the controls, and the eggs were often clustered as if they were sticky (Rugh, 1953b). Lower exposures did allow a higher percentage of cleavage, and further development, but the 50,000 r level of exposure was chosen since the low level of 13% cleavage could be improved if there were any protection. In 13 separate sets of data, involving thousands of eggs, only 5 or 6 exposed to 50,000 r were able to achieve the pluteus

stage, and not many more survived the process of gastrulation. This made the end point of development, following 50,000 r gamma rays, clear-cut.

The AET was neutralized to pH 7.3–7.6 but was found to be toxic for *Arbacia* eggs in concentrations comparable to those used for mammals. Membranes were elevated, asters were formed, and cleavages did occur in concentrations up to 60 mgm.% (2% cleavages). However, since as little as 5 mgm.% AET in sea water destroyed the developing *Arbacia* eggs some time after fertilization and early cleavage, if left in the solution, it was decided to use 3 mgm.%, which allowed 97% of the unirradiated eggs to be fertilized and cleave normally, both with respect to time and form. A lower concentration of 1 mgm.% allowed *Arbacia* eggs to be fertilized and to develop, similarly with the controls, through the pluteus stage. There appeared to be no evidence that this concentration was in any way toxic. The concentration of 3 mgm.% was therefore chosen for use prior to and during irradiation since, in a few experiments, this concentration did appear to have a very slight delaying effect on the time of the first cleavage if the eggs were left in the AET solution. This concentration was therefore considered to be at the threshold level of toxicity. The crucial experiment, based upon these empirical findings with irradiation and AET concentrations, was exposure of the eggs for 10 minutes prior to and 10 minutes during gamma irradiation (50,000 r) to the 3 mgm.% solution of AET, and then transferring them to filtered sea water for fertilization and development. Continuing exposure of the irradiated eggs to AET allowed slight improvement in the time and percentage of cleavage after fertilization; only 20% became ciliated blastulae and all were dead by 48 hours. Thus, it seemed that the optimum conditions involved return of the irradiated eggs from the AET to filtered sea water for fertilization and development.

The data of the final experiment alone will be given since they followed the general pattern of the prior experiments and had higher counts for each of the variables, of 400 cells per sample.

The data of the table above substantiate Henshaw (1932, 1940) and Henshaw and Francis (1936), who report that a delay in fertilization of irradiated and unfertilized eggs of *Arbacia* allows some of them to "recover" so that the retardation in the initial cleavage normally caused by irradiation is somewhat nullified. The percentage difference is not great, after 50,000 r exposure, but the eggs irradiated at the beginning of the series started to cleave at a shorter elapsed time interval than those irradiated at the end of the series, due to the recovery phenomenon of Henshaw. Eggs in 12 mgm.% AET showed reduced percentage of cleavage throughout, and by 24 hours virtually all were dead. Likewise, those irradiated in 12 mgm.% but returned immediately to sea water for fertilization and development showed no "protection" by this agent, and all were dead by 24 hours. In 3 mgm.% AET the results were comparable to the controls, indicating no significant delay or deleterious effect on cleavage or development. When eggs were irradiated in 3 mgm.% AET and immediately returned to sea water for fertilization, there was no improvement in cleavage time in relation to those irradiated without the drug. By 24 hours only 30% became ciliated, and were retarded in development. As expected, 1 mgm.% AET had no effect on either fertilization or development. Thus, while AET in all concentrations used allowed fertilization of the treated eggs, and 3 mgm.% was definitely non-toxic, no concentration used prior to and during

irradiation had any "protective" effect, either on the time of the initial cleavage, gastrulation or later development. While 3 mgm.% AET allowed unirradiated eggs to be fertilized and develop (by 48 hours) to motile plutei, no eggs irradiated in AET to 50,000 r gamma rays ever reached the pluteus stage, or even gastrulation.

TABLE I

Cleavage % (400 eggs) at intervals after fertilization

Conditions	60′	80′	100′	120′	24 hrs.	48 hrs.
Controls	74%	92%	94%	99%	Cil. gastrula	Norm. plutei
50,000 r gamma rays	0	0	2	11	30% cil. blastula	0.1% cil. blastula, no plutei
12 mgm. % AET only	25	32	36	49	30% alive, retarded	20% alive, abnormal, retarded
12 mgm. % AET + 50,000 r + sea wate	0	0	1	4	99% dead	0.1% cil. blastula
3 mgm. % AET only	72	90	95	99	Cil. gastrula	Norm. plutei
3 mgm. % AET + 50,000 r + sea water	0	0	1	7	30% cil. blastula	0.1% cil. blastula, no plutei
3 mgm. % AET + 50,000 r + AET	0	0	9	21	30% cil. blastula	100% dead
1 mgm. % AET only	69	90	96	99	99% cil. gastrula	Norm. plutei
50,000 r gamma rays*	0	0	0	2	10% cil. blastula abnormal	100% dead

*Unfertilized eggs were irradiated at the beginning of the series and another group at the end (30 minutes later) but all were fertilized simultaneously so that the first group had 30 minutes "recovery" time, due to delay in fertilization. The effect of the delay is reflected in the slight difference in cleavage data.

DISCUSSION

"Protection" in radiobiology is really a misnomer. Its limitations should always be clearly defined by the user. Even among mammals there are only a few agents which, if present in the body at the time of exposure, will provide greater tolerance of whole body exposure to ionizing radiations, resulting in a higher LD/50/30 (*i.e.*, lethal dose to 50% of exposed animals in 30 days). But this is *survival*, and, to that extent, protection against death. The tendency is to assume that all animals that survive a 30-day post-irradiation period have "recovered" from any and all ill-effects of the exposure and are as normal as they were prior to the exposure. This is simply not true. Whole body exposure takes a toll which can be expressed in a variety of sequelae, and some effects are irrevocable, irreparable. This is not to belittle the possible clinical importance of devising means to extend survival

to a larger population of exposed individuals, no matter how they fare in their survival. But we must not be blinded to the sequelae of such exposure and survival.

The mechanism of "protection" by AET solutions has been elusive to many investigators. It has been thought to function through aiding in hematopoietic recovery, but the same agents which seem to do this are deleterious if given after irradiation. It has been suggested that AET might help to remove toxic radicals before they can injure normal cells, or in some way to actually stimulate cell proliferation in specific tissues. While all theories have been vague, it has been presumed that any "protection" should ultimately be demonstrable at the cellular level. Tied in with protection of the adult mammal (Khym *et al.*, 1957) is the demonstrated fact that genes and chromosomes are particularly radiosensitive. Recently, Mittler (1963) found that neither AET nor MEA had any protective effect against induced mutations or translocations in chromosomes of *Drosophila* spermatocytes or spermatids. But AET has been the most effective agent with mammals so that this present study seemed imperative.

There is a wide species variation in the toxicity levels and the responses to AET (Colclough and Hanna, 1963), ranging from 10–20 mg./kg. given orally to man, to 640 mg./kg. injected into mice (Doherty, personal communication). Its chemistry and degradation products are well documented by Doherty and his co-workers. Since it is not a highly stable compound, except under rather rigid laboratory conditions, it may not prove to be as universally useful as might be desired. Nevertheless, among the hundreds of chemical agents tried it now appears to be the most effective for most mammals.

The *Arbacia* egg is so well known, so dependable in its reactions to almost all environmental variables, that it proves to be an ideal haploid cell on which to test the efficacy of any possible radioprotective agent. The several criteria of effect are: fertilizability, membrane elevation, aster formation, cleavage time, cleavage form, blastula-gastrula transition, and plutei formation. Each successive criterion is the more sensitive, as development proceeds, and if the pluteus stage is attained one may consider survival to be quite complete, at least as complete as 30-day survival for the mammal.

Ionizing radiations (x-rays) have been shown to have a direct effect on the cleavage mechanism of *Arbacia* so that, with increasing exposures (within limits) there is increasing delay in the time of the first cleavage (Henshaw, 1940). Recently Rao (1963) demonstrated that this was due to chromosome condensation interfering with the mechanism of mitosis. Time appears to heal, somewhat, irradiation damage because a delay in the fertilization of irradiated eggs reduces the effect of that irradiation on the initiation of the first cleavage. This was originally referred to as "recovery" (Henshaw, 1940), but was more recently shown not to be full recovery but rather a return toward the normal cleavage times (Rugh and Wolff, 1956; Rugh, 1958), with the ultimate embryonic death unaltered. This recovery of cleavage time was nevertheless of interest in view of the finding (Rugh, 1950) that ionizing radiations so affect (meiotic) chromosomes that they become sticky and are permanently clumped. Delay in fertilization could hardly disentangle fused chromosomes, although such delay could allow time for the re-fusion of fragmented chromosomes. Henshaw (1938) long ago showed that the delay in

cleavage time seen in irradiated eggs was not seen in the enucleated fragments of *Arbacia* eggs, so that this phenomenon is directly related to chromosome effects. Apparently it is the early prophase stage which is so affected, and such delay is not carried over into subsequent cleavages (Yamashita *et al.*, 1939).

In order to avoid confusing any possible effect of AET with the "recovery" in normal cleavage time due simply to a delay in fertilization of the egg, control-irradiated eggs were provided at the beginning and at the end of each experimental series. Thus, the maximum "recovery" of cleavage time would be demonstrated by the first control-irradiated eggs (which had maximum delay in fertilization) while the maximum damage from irradiation would be shown in the cleavage percentages of the final group, fertilized immediately after irradiation. Since the temperature was uniform for any set of variables of any single experiment, this factor could be ruled out.

AET was positively *not* protective to the egg of *Arbacia*, using any of the criteria listed above. This does not rule out the possibility that some other agent(s) might be "protective" in one or another sense. Certainly the mechanism of the so-called "protection" need not be identical for all cells, tissues, organs or organisms, but this agent, so useful with the mammal, is totally without benefit to the gamma-irradiated haploid egg cell of *Arbacia*, prior to fertilization.

Summary and Conclusions

1. AET (S,2-aminoethylisothiourea-Di-HBr) was used in a concentration just below the threshold toxicity level, to determine whether it might afford any radio-protection for the haploid *Arbacia* egg exposed to 50,000 r gamma radiation prior to fertilization with normal sperm.

2. *Arbacia* eggs could be fertilized in 3 mgm.% AET and would cleave and develop, both in time and manner comparable to the controls in pure sea water. Toxicity was indicated above 5 mgm.%, particularly after irradiation.

3. *Arbacia* eggs exposed to 50,000 r gamma rays showed a delay in the initiation of the first cleavage with ultimate cleavage reaching only 11% and abnormalities reaching 18%. Not a single egg so exposed ever reached the pluteus stage. The delay in the initiation of the first cleavage was also reduced by a delay in fertilization, and the percentage of ultimate cleavage was improved.

4. The optimum conditions provided were: Exposure to 3 mgm.% AET in sea water for 10 minutes prior to and 10 minutes during gamma irradiation to 50,000 r, and yet this allowed no improvement in cleavage time, degree of membrane elevation, or development. Not a single egg thus treated reached either the pluteus or gastrula stages.

5. It is concluded that while AET has proven to be radioprotective for the adult mammal, this protection (survival) may not be effected through individual cells but through tissue or organ regeneration. However, extrapolation is always hazardous and AET may be cell- or species-specific. The haploid *Arbacia* cell (cytoplasm and nucleus) is not subject to any protective action from AET.

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